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# Comparative analysis of $\alpha$ - and $\beta$ -thujone in the essential oil and supercritical CO<sub>2</sub> extract of sage (*Salvia officinalis* L.)

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## Abstract

*Salvia officinalis* L. (Sage) is an important industrial plant used both for food and pharmaceutical purposes. The terpene fraction of this plant, which contains thujones and other mono and sesquiterpenoids, is responsible for many of its therapeutic and culinary properties. We used two extraction methods [hydrodistillation (HD) to obtain the essential oil (EO), and supercritical CO<sub>2</sub> extraction (SFE)] to analyze by gas chromatography–mass spectrometry (GC–MS) the terpene fraction extracted from sage dried leaves.  $\alpha$ -Thujone,  $\beta$ -thujone and other oxygenated monoterpenes (1,8-cineole, linalool, camphor, borneol and bornyl acetate) as well as hydrocarbon ( $\beta$ -caryophyllene and  $\alpha$ -humulene) and oxygenated sesquiterpenes (caryophyllene oxide, viridiflorol, humulene epoxide II and III) were found. The EO contained a significantly ( $p < 0.05$ ) higher percentage of 1,8-cineole (10.4%),  $\alpha$ -thujone (17.3%) and camphor (29.2%), whereas supercritical fluid (SF) extracts contained a significantly higher percentage of borneol (8.4%), bornyl acetate (2.2%),  $\alpha$ -humulene (6.4%), viridiflorol (22.1%), humulene epoxide II and III (2.4% and 0.4%), and some unidentified sesquiterpene alcohols. Both EO and SF extracts contained equal amounts of  $\beta$ -thujone (4.8%) and  $\beta$ -caryophyllene (~7%). Our results show that HD of EO is a more efficient and economic method for  $\alpha$ - and  $\beta$ -thujone extraction.

## Keywords

- *Salvia officinalis*,
- Lamiaceae,
- essential oil,
- supercritical CO<sub>2</sub> extraction and hydrodistillation,
- $\alpha$ -thujone,
- $\beta$ -thujone,
- GC–MS

## Introduction

*Salvia officinalis* L. (sage) is an odorous small perennial shrub native to the Mediterranean region and it is largely cultivated for culinary and medicinal purposes ([1](#)). Terpenoids and phenolics have been identified as the two major typical sage secondary metabolites. Among the terpenoids, volatile oils have been largely investigated ([2](#), [3](#)). Sage essential oils (EO) have been studied for their toxicity ([4](#), [5](#)) and because of their antimutagenic ([6](#)), antimicrobial ([7–9](#)), antiviral ([10](#)), preservative ([11](#), [12](#)), immunomodulatory ([13](#)), antioxidant ([1](#), [14](#)), larvicidal ([15](#)) and anticancer ([16](#)) properties.

Variations in the compositions of the sage terpene fraction are considerable depending on the quality of the plant material as well as to the methods used for extraction. For instance, EO usually contains a reduced amount of the less volatile compounds than in the case of solvent extraction ([17](#)). Moreover, the use of hydrodistillation (HD) has often been discussed very critically because of

either transformation processes of genuine aroma-active compounds due to the influence of heat, steam and pH or highly volatile loss (18). Besides HD, supercritical fluid extraction (SFE) has been used to characterize volatile fractions from aromatic plants (19–23). SFE has been used to obtain sage fractions with increased antioxidant activity (24, 25), higher oxygenated monoterpene yields (26) and a wider spectrum of phytochemicals compared with Soxhlet, solvent extraction or HD (27). SFE is performed generally using carbon dioxide (CO<sub>2</sub>) for its moderately low critical pressure (7.4 MPa) and temperature (32°C), low toxicity, high purity at relatively low cost and easy removal from the extract. Since supercritical CO<sub>2</sub> has a polarity similar to liquid pentane, it is suitable for extraction of lipophilic compounds such as terpenes (28).

Thujone is a natural monoterpene also associated with sage. There is currently a heated debate on the toxicity of thujones (29–31). Since one of the major dietary contribution of thujones appears to derive from sage, the purpose of this research is a comparative analysis of  $\alpha$ - and  $\beta$ -thujone extraction methods, namely HD and SFE, to explore the better method to analyze their presence.

## Experimental

### Plant material

*Salvia officinalis* L. dried leaves were purchased from the Pharmaceutical Laboratory Labofarm (Starogard Gdanski) in Poland. Samples were ground using an electrical blender, stored in closed containers at 5°C in the dark and then used for both HD and SFE.

### Essential oil hydrodistillation and supercritical CO<sub>2</sub> extraction

Twenty grams of dried sage leaves were hydrodistilled for 2 hours by using a modified Clevenger apparatus as described previously (19). The EO was collected, weighed and then stored in a refrigerator at –20°C before gas chromatography–mass spectrometry (GC–MS) analysis.

The SFE of dried sage leaves (320 g) was carried out with a Waters SFE-2000F unit (Waters SFC Division, Pittsburg, PA, US) in a 2-L extractor. Food-grade (99.9% v/v purity) CO<sub>2</sub> (Air Liquide, Italy) was delivered for 90 minutes at a flow rate of 6 kg/hour and was compressed to the extraction pressure of 25 MPa by an air-driven liquid pump after cooling. The CO<sub>2</sub> was delivered to the extractor via a heat exchanger to maintain the extraction temperature at 60°C. At the same time, 95% (w/w) ethanol (Carlo Erba, Italy) was added using a co-solvent pump at the concentration of 2% (w/w). Two cyclonic separators (500 mL each) were used in order to obtain two different extracts: (5.5 MPa, 25°C) waxes and other heavy components in the first separator (2 MPa, 20°C), the terpenic fraction used for this study in the second separator. The latter was weighed and stored in a refrigerator at –20°C before GC–MS analysis.

### Gas chromatography and mass spectrometry

The EO and the supercritical fluid (SF) extract were analyzed by gas chromatography (Agilent Technologies, mod. 6890N) coupled with mass spectrometry (Agilent technologies, mod. 5973A). Compounds were separated on a Zebron ZB-5MS (mod. 7HG-G010-11, Phenomenex, USA) capillary column (stationary phase: 95% polydimethyl siloxane–5% diphenyl, 30 m length, 250  $\mu$ m internal diameter, 0.25  $\mu$ m film thickness) with the following temperature program: 60°C for 5 minutes followed by a temperature rise at a 3°C/minute rate to 270°C (held for 5 minutes). Carrier gas was He with a constant flow of 1 mL/minute, transfer line temperature to MSD was 280°C, ionization energy (EI) 70 eV and full scan range 50–300  $m/z$ .

## Component identification

Separated compounds were identified by pure standard comparison, by comparison of their mass spectra and linear retention indexes (Kováts index) with those of reference substances and by comparison with the NIST mass spectral search software v2.0 using the NIST 98 library. Linear retention indices (RI) were calculated against a C<sub>8</sub>–C<sub>20</sub> *n*-alkanes mixture. Literature indices were taken from Adams (32).

## Statistical analysis

The overall data sets are expressed as mean values of at least three biological replicates. Three technical replicates were run for each biological replicate. Analysis of variance (ANOVA) and Tukey–Kramer’s HSD test ( $p < 0.05$ ) were used to determine significant differences among extractions using the SYSTAT 10 software.

## Results and discussion

This study aims to compare two extraction methods for terpene analysis in the food and pharmaceutical plant *S. officinalis* L. For this reason, we extracted EO from dried sage leaves by HD and also obtained SF extracts, both subjected to GC–MS analyses.

The yield of EO was 90 ( $\pm 7.5$ ) mg/kg leaf dry weight, whereas the yield of SF extracts was 97 ( $\pm 3.7$ ) mg/kg leaf dry weight. A statistical analysis showed no difference ( $p > 0.05$ ) between yields of the two extraction methods.

In general, the volatile fraction of sage was characterized by the presence of several monoterpene hydrocarbons ( $\alpha$ -pinene, camphene, sabinene,  $\beta$ -pinene, myrcene,  $\alpha$ -terpinene, *o*-cymene, limonene,  $\gamma$ -terpinene and  $\alpha$ -terpinolene); oxygenated monoterpenes (1,8-cineole, linalool,  $\alpha$ -thujone,  $\beta$ -thujone, camphor, menthol, thymol, borneol and bornyl acetate) as well as hydrocarbon ( $\beta$ -caryophyllene, ledene,  $\delta$ -cadinene and  $\alpha$ -humulene) and oxygenated sesquiterpenes (caryophyllene oxide, globulol, viridiflorol, humulene epoxide II and III and some unidentified compounds), in agreement with literature data (1, 33–38).

Table 1 reports the volatile content (expressed as relative area percentage) of EO (Figure 1) and SF extract (Figure 2) together with their linear retention indices (LRI), calculated against a C<sub>8</sub>–C<sub>20</sub> *n*-alkanes mixture and from literature data (32).

Table 1. Essential oil (EO) and supercritical CO<sub>2</sub> extract (SFE) of *Salvia officinalis* L.

Figure 1. Total ion chromatogram on a Zebron ZB-5MS capillary column (stationary phase: 95% polydimethyl siloxane–5% diphenyl, 30 m length, 250  $\mu$ m internal diameter, 0.25  $\mu$ m film thickness) of *Salvia officinalis* essential oil. **1:**  $\alpha$ -pinene, **2:** camphene, **3:** sabinene, **4:**  $\beta$ -pinene, **5:** myrcene, **6:**  $\alpha$ -terpinene, **7:** limonene, **8:** 1,8-cineole, **9:**  $\gamma$ -terpinene, **10:**  $\alpha$ -terpinolene, **11:** linalool, **12:**  $\alpha$ -thujone, **13:**  $\beta$ -thujone, **14:** thujyl alcohol, **15:** camphor, **16:** borneol, **17:** menthol, **18:** bornyl acetate, **19:** thymol, **20:**  $\beta$ -caryophyllene, **21:**  $\alpha$ -humulene, **22:** ledene, **23:**  $\delta$ -cadinene, **24:** caryophyllene oxide, **25:** viridiflorol, **26:** humulene epoxide II.

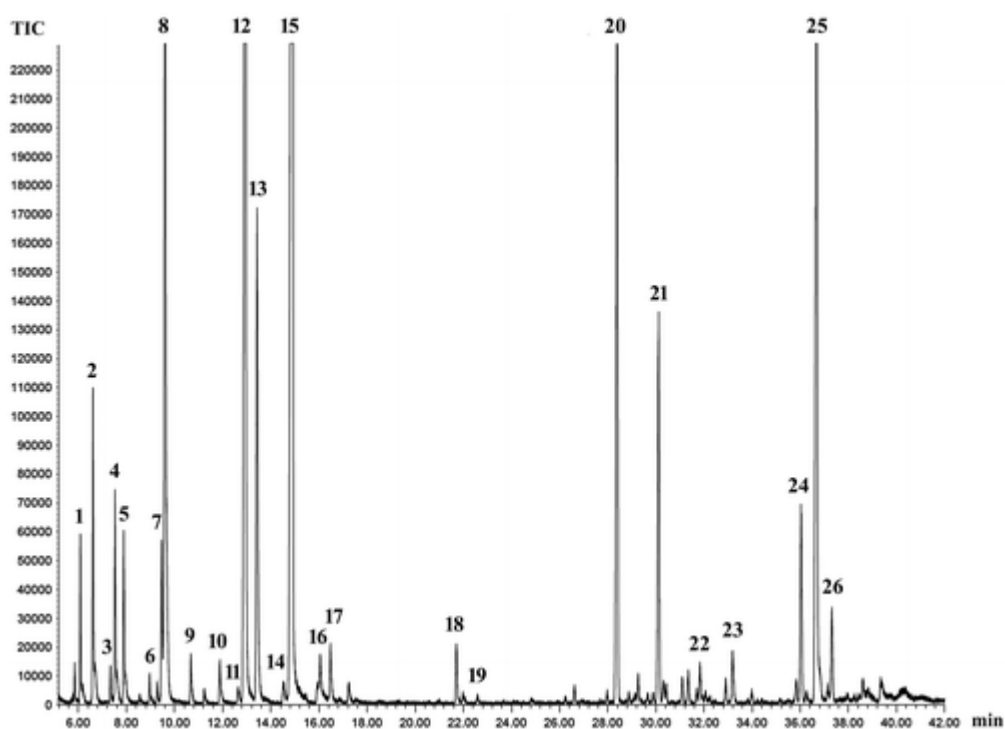
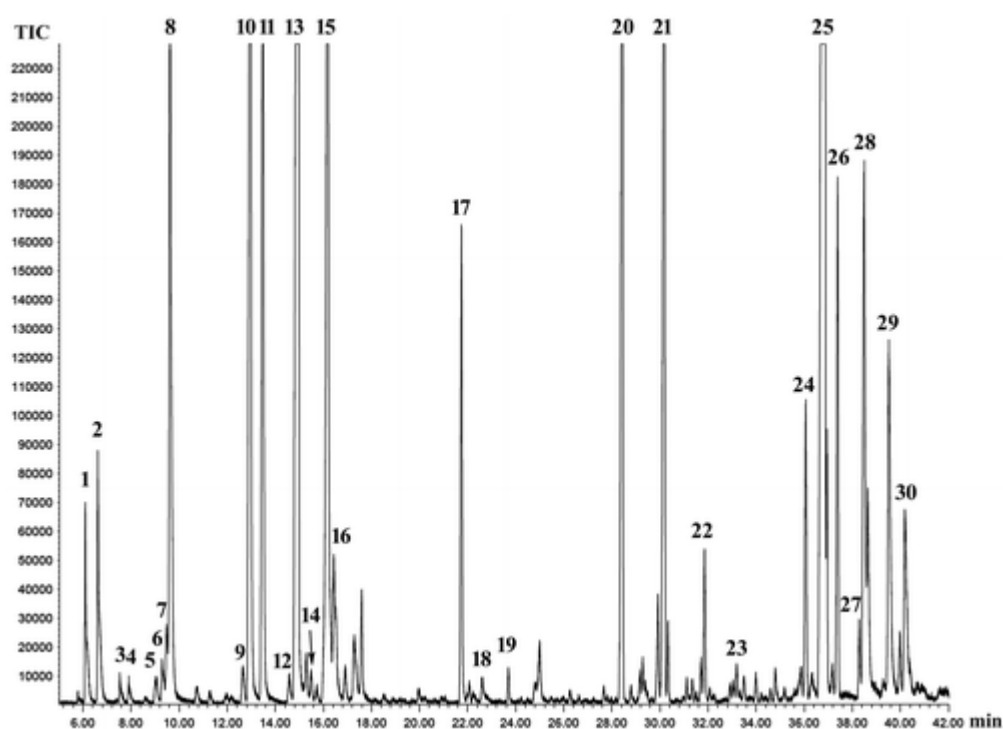


Figure 2. Total ion chromatogram on a Zebron ZB-5MS capillary column (stationary phase: 95% polydimethyl siloxane–5% diphenyl, 30 m length, 250  $\mu\text{m}$  internal diameter, 0.25  $\mu\text{m}$  film thickness) of *Salvia officinalis* supercritical  $\text{CO}_2$  extract. **1:**  $\alpha$ -pinene, **2:** camphene, **3:**  $\beta$ -pinene, **4:** myrcene, **5:**  $\alpha$ -terpinene, **6:** *O*-cymene, **7:** limonene, **8:** 1,8-cineole, **9:** linalool, **10:**  $\alpha$ -thujone, **11:**  $\beta$ -thujone, **12:** thujyl alcohol, **13:** camphor, **14:** pinocamphone, **15:** borneol, **16:** menthol, **17:** bornyl acetate, **18:** thymol, **19:** myrtenyl acetate, **20:**  $\beta$ -caryophyllene, **21:**  $\alpha$ -humulene, **22:** ledene, **23:**  $\delta$ -cadinene, **24:** caryophyllene oxide, **25:** viridiflorol, **26:** humulene epoxide II, **27:** humulene epoxide III, **28:** unknown sesquiterpene alcohol, **29:** unknown sesquiterpene alcohol, **30:** unknown sesquiterpene alcohol. (For the MS spectra of compounds **28**, **29** and **30**, see Supplementary Figure S1).



The content of  $\alpha$ -thujone showed a significantly ( $p < 0.05$ ) higher percentage in the EO (17.3%), when compared with the SF extract (7.5%), whereas the percentage of  $\beta$ -thujone was not significantly different between the EO (4.8%) and SF extract (4.9%).

Considering monoterpenes, the EO showed a significantly ( $p < 0.05$ ) higher percentage of camphene, sabinene,  $\beta$ -pinene, myrcene, limonene,  $\gamma$ -terpinene,  $\alpha$ -terpinolene, 1,8-cineole and camphor, when compared with the SF extract (Table 1, Figures 1 and 2). However, the SF extracts showed a significantly higher percentage of borneol, menthol, bornyl acetate and myrtenyl acetate.

The sesquiterpene percentage of the SF extract was significantly higher than that of the EO. In particular, higher SF extract percentages were found for  $\alpha$ -humulene, viridiflorol, humulene epoxide II, humulene epoxide III and three unidentified sesquiterpene alcohols (see Supplementary Figure S1 for MS spectra), when compared with the EO (Table 1, Figures 1 and 2). The percentage of  $\beta$ -caryophyllene, ledene and caryophyllene oxide was not significantly different in the SF extract and EO.

During the past few years, several papers described SFE of sage from different origin (27 and references cited therein). Our data are in agreement with a reduced extraction of some monoterpene oxygenated compounds and an increase of sesquiterpenes (24–27, 39–41). However, most of the SFE-related literature is currently reporting data from laboratory extractors with extraction volumes far from industrial process scales. Our 2-L system allowed extracting sage terpenoids in conditions closer to industrial process, thus producing results that are more reliable and realistic.

In conclusion, the results here reported shown that HD is the most effective and economical method of volatile EO extraction from the determination of  $\alpha$ -thujone and  $\beta$ -thujone in sage dried leaves, with SF extracts showing similar ( $\beta$ -thujone) and in some case lower ( $\alpha$ -thujone) ability to extract the main sage bioactive compounds.

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